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COURTESY COPY OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT WITH ANNEXES CONTAINING NEW PAGES 8, 9, 103, 112, 119, 120, 126, 127 and 129-131 OF THE SPECIFICATION TO REPLACE ORIGINAL PAGES 8, 9, 103, 112, 119, 120, 126, 127 and 129-131 OF THE SPECIFICATION; NEW CLAIMS 1-11 TO REPLACE ORIGINAL CLAIMS 1-57 AND DRAWING SHEETS 38/49-49/49 TO REPLACE ORIGINAL DRAWING SHEETS 38/49-49/49 FOR EXAMINATION IN THIS CASE

PATENT COOPERATION TREATY

PLOUGMANN VINGTOFT & PARTNERS

14 NOV. 2001

From the INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT JSK/MSN

To:

PLOUGMANN VINGTOFT & PARTNERS A/S
Sankt Annae Plads 11
P.O. Box 3007
DK-1021 Copenhagen K
DANEMARK

corrected version

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	12.11.2001
Applicant's or agent's file reference 23725 PC 1	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK00/00425	27/07/2000	27/07/1999

Applicant
HEMEBIOTECH A/S et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Exner, K Tel.+49 89 2399-7826

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
23725 PC 1		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK00/00425	27/07/2000	27/07/1999
International Patent Classification (IPC) or national classification and IPC		
A61K38/00		
Applicant		
HEMEBIOTECH A/S et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 25 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
27/02/2001	12.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Fayos, C Telephone No. +49 89 2399 2180

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/DK00/00425

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-7,10-102,104-111, 113-118,121-125, 128,132-138	as originally filed			
8,9,103,112,119, 120,126,127, 129-131	as received on	13/10/2001	with letter of	10/10/2001

Claims, No.:

1-11	as received on	13/10/2001	with letter of	10/10/2001

Drawings, sheets:

1/49-37/49	as originally filed			
38/49-49/49	as received on	13/10/2001	with letter of	10/10/2001

Sequence listing part of the description, pages:

1-11, filed with the letter of 19.09.01

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
see separate sheet

6. Additional observations, if necessary:
see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 6 and 9.

because:

☒ the said international application, or the said claims Nos. 6 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 9 are so unclear that no meaningful opinion could be formed (*specify*):
see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-5, 7-8 and 10-11
	No:	Claims	-
Inventive step (IS)	Yes:	Claims	1-5, 7-8 and 10-11
	No:	Claims	-
Industrial applicability (IA)	Yes:	Claims	1-5, 7-8 and 10-11
	No:	Claims	-

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

Re Item I
Basis of the report

1- An amended set of claims has been enclosed to the applicant's letter dated 10.10.01. However, the mentioned set of claims wherein the amendments are indicated was not enclosed in said letter.

1.1- Claims 1-53 have been deleted.

1.2- New claim 9 has been introduced to the rhPBGD produced by the method of any of claims 2-6.

1.3- In claim 2 b), a reference to the introduction of the production strain according to claim 1 has been added.

1.4- New claim 6 is based on old claim 44. However, it does not meet the requirements of Article 34(2)(b) PCT (see item III 3- below).

Re Item II
Priority

2- The priority date (27.07.1999) appears to be valid for the subject matter claimed. Hence, D4 is not prior art in this case. D4 cited in the international search report will probably become relevant in the European phase.

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

3- The amendments filed with the letter dated 10.10.01 introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT.

The amendment concerned is that of claim 6.

No basis could be found in the application as originally filed for "a method for the preparation of rhPBGD" wherein the PBGD "is recombinant human PBGD based on any of Seq. ID NO 3 (clone PBGD 1.1) and Seq. ID NO 4 (non-erythro PBGD 1.1.1).

2.1- Claims 1-5 and 7-11 meet the requirements of Article 34(2)(b) PCT.

2.2- The corrections made to the description of the present application and the drawings do not contravene Article 34(2)(b) PCT.

The applicant's observations (10.10.01) submitted with the amended claims have been considered.

5- Claim 9 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined.

Claim 9 attempts to define the subject-matter in terms of the result to be achieved (i. e. "able to lower the levels of PBG and ALA in mice during an acute attack of porphyria in a transgenic mouse model where the PBGD gene has partially been knocked-out") which merely amounts to a statement of the underlying problem "a rhPBGD which is able to lower the levels of PBG and ALA in mice during an acute attack of porphyria in a transgenic mouse model where the PBGD gene has partially been knocked-out" . The technical features necessary for achieving this result are missing. Therefore, no opinion on novelty, inventive step and industrial applicability will be formulated with regards to the subject matter of claim 9.

5.1- A rhPBGD produced by the method of any of claims 2-6 could be considered as being novel over the prior art cited in the search report.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

6- Reference is made to the following documents:

D1: GROSS U ET AL: 'Haem precursors and porphobilinogen deaminase in erythrocytes and lymphocytes of patients with acute intermittent porphyria' CELLULAR AND MOLECULAR BIOLOGY,US,TARRYTOWN, NY, vol. 43, no. 1, 1 February 1997 (1997-02-01), pages 29-35, XP002082339 ISSN: 0145-5680

D2: SASSA S: 'Diagnosis and therapy of acute intermittent porphyria' BLOOD REVIEWS,GB,EDINBURGH, vol. 10, no. 1, 1 March 1996 (1996-03-01), pages 53-58, XP002082340

D3: GRANDCHAMP B: 'Acute intermittent porphyria' SEMINARS IN LIVER DISEASE,DE,STUTTGART, vol. 18, no. 1, 1 January 1998 (1998-01-01), pages 17-24, XP002082341

D4: see item VI

6.1- Additional document:

D5: MOLECULAR CELL BIOLOGY (Third edition- 1995) p 299-300

NOVELTY - Art. 33 (1) and (2) PCT

7- Claims 1-5, 7-8 and 10-11 appear to be novel in the light of the prior art cited in the search report:

7.1- The novel features are:

- a production strain of rhPBGD as in claim 1,
- a method for the preparation of rhPBGD as in claims 2-5,
- an expression plasmid as in claim 7,
- a DNA fragment as in claim 8, and
- a rhPBGD as in claim 10 or claim 11.

INVENTIVE STEP - Art. 33 (1) and (3) PCT

8- Claims 1-5, 7-8 and 10-11 appear to be inventive over the cited prior art for the reasons stated below:

8.1- The claims have been reformulated to define the problem to be solved to be to provide a production strain and methods of preparing rhPBGD by use of said production strain in order to produce sufficient quantities of enzyme, i. e. functional recombinant human PBGD, to make enzyme replacement therapy possible.

The objective problem posed in the present application is to provide means for large scale production of rhPBGD.

The solution proposed is to provide a production strain as in claim 1 of the present application.

8.2- The prior art documents cited in the search report, alone or combined, fail to describe a large scale production of rhPBGD.

In fact, the present application provides a production strain which is particularly suitable for large-scale production of the enzyme since it has been engineered not to express the endogenous Phrophobillinogen Deaminase of E. coli. In order to provide this production strain, it was necessary to clone a functional enzyme selected from a large group of PBGD variants. In view of the large number of mutations and polymorphisms, it was not straightforward which one to select and whether it would be suitable for the purpose.

Thus, in real world context, it would not have been obvious for the skilled man to provide a production strain as in claim 1 of the present application.

Hence, claims 1-5, 7-8 and 10-11 can be considered as being inventive.

It is well known (see any of D1-D3) that AIP is an autosomal dominant disorder resulting from a patrial porphobilinogen deaminase (PBGD) deficiency. Furthermore, the gene coding for PBGD has been identified, its cDNA has been cloned and the mutations which cause AIP are also known (see D2 and D3).

It would therefore be obvious for the skilled man, to compensate the deficiency in PBGD in a subject by administrating PBGD (directly, or by means of gene therapy) and hence normalize the levels of PBGD in said subject.

However, there is no suggestion in any of the prior art documents, taken alone or in combination to provide a production strain as in claim 1 of the present application.

INDUSTRIAL APPLICABILITY - Art. 33 (1) and (4) PCT

9- Claims 1-5, 7-8 and 10-11 appear to be industrially applicable.

Re Item VI

Certain documents cited

10- Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 99 37325	29.07.1999	27.01.1999	27.01.1998 30.12.1998

CLAIMS

1. A production strain of rhPBGD obtained by use of the DNA fragment, EcoR I - Hind III linear fragment as shown in SEQ ID NO 2 to obtain hemC-deletion in the host JM105-H-R6-C by homologous gene replacement and transforming the resulting strain with the expression plasmid pExp1-M2-BB as shown in SEQ ID NO:1 to yield the final production strain PBGD which is free from production of PBGD of non human origin (Accession No 12915).

2. A method for the preparation of rhPBGD by a method comprising

a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding PBGD;
b) transforming the production strain according to claim 1 with the vector;
c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence;
d) recovering the expression product from the culture.

3. A method according to claim 2 further comprising a fermentation step.

4. A method according to claim 2 or 3 further comprising a purification step.

5. A method according to claim 4 wherein the purification is performed with a His-Tag (rhPBGD-His).

6. A method according to any of claims 2-5, wherein the PBGD is recombinant human PBGD based on any of Seq. ID NO 3 (clone PBGD 1.1) and Seq. ID NO 4 (non-erythro PBGD 1.1.1).

7. An expression plasmid pExp1-M2-BB as shown in Seq. ID NO 1 for use in the expression of rhPBGD in E. coli.

8. A DNA fragment, EcoR I - Hind III linear fragment as shown in Seq. ID NO 2, capable of obtaining hemC-deletion in a host.

9. A rhPBGD produced by the method of any of claims 2-6 and able to lower the levels of PBG and ALA in mice during an acute attack of porphyria in a transgenic mouse model where the PBGD gene has partially been knocked-out.

10. A rhPBGD having a stability of at least 6 weeks at 20°C, such as for at least 7 weeks, preferably for 8 weeks.

11. A rhPBGD having a stability resulting in a decrease in activity of less that 10% per month, such as less than 5%.

Figure 16: Chromatography on Butyl-Sepharose 4 FF

Figure 17: Circular map of rhPBGD-His expression plasmid pExp2

Figure 18: PBGD reaction mechanism

Figure 19: DEAE chromatography elution profile

Figure 20: SDS-PAGE gel of DEAE eluates

Figure 21: Cobalt chromatography elution profile

Figure 22: SDS-PAGE gel results of cobalt eluates

Figure 23 and

Figure 24: Ilustrate numbers in diagrams (Table19). The expression of PBGD in HeLa cells was increased up to 475 times from the basal activity and in NIH 3T3 cells up to 11 times.

Figure 25: Comparison of fermentations PD05 and PD06 with strain PBGD-2

Figure 26: Comparison of fermentations PD09, PD11 and PD12

Figure 27: Comparison of fermentations PD09, PD11 and PD12 with strain PBGD-1.

Figure 28: Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.

Figure 29: Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2

Figure 30: Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.

Figure 31: Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.

Figure 32: Comparison of fermentations PD19, PD1501 and PD1502

Figure 33:: Comparison of fermentations PD19, PD1501 and PD1502 with strain PBGD-2.

Figure 34:

Figure 35: Stability studies: Single use aliquots of extract were routinely taken out of the freezer (-20°C) and the rhPBGD-activity was measured and plotted over time.

Figure 36.Description of oligos used for PCR amplification.

Figure 37 A, and B:Strategy for PCR cloning of ALAD

Figure 37C. pBlue AlaD Restriction map.

Figure 38: Plasma levels of rhPBGD following administration to mice. 50 µg rhPBGD (2,3-2,8 mg/kg)

Figure 39: PBGD enzymatic activity in plasma following rhPBGD administration to mice

Figure 40: The urinary content of PBG and ALA in AIP-mouse treated with phenobarbital.

Figure. 41shows the urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD.

Figure 42: Shows the grip strength analysis in control and AIP-mice

Figure 43: Rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Figure 44. Enzyme concentration over 8 weeks at 40°C measured by HPLC. A decrease from 2 mg/ml to 0,5 mg/ml and 8 mg/ml to 2,5 was detected.

Figure 46. Enzyme specific activity measured during 8 weeks at 40°C. The activity was measured using the enzyme activity assay and the protein concentration was measured using HPLC.

Figure 45. The enzyme activity measured over 8 weeks at 40°C. A significant decrease over the first week was seen for the high concentration sample, 1b. After two weeks the decrease rate was the same for all samples.

Figure 47. rhPBGD concentration over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. The measurement was performed using HPLC

Figure 48. rhPBGD activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling.

Figure 49. rhPBGD specific activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. Measurements were performed using enzyme activity assay and HPLC.

Figure 50. rhPBGD concentration measured over 8 weeks using BCA.

Figure ~~51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.~~

Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Figure 52. The specific rhPBGD activity measured using the enzyme activity assay and BCA protein concentration assay. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Sequence list:

Seq. ID NO 1: Sequence of the expression plasmid pExp1-M2-BB

Seq. ID NO 2: Sequence of the EcoR I - Hind III linear fragment used for transformation in the hemC disruption strategy

Seq. ID NO 3: Sequence of the erythropoietic form (PBGD 1.1)

Seq. ID NO 4: Sequence of the non-erythropoietic form (PBGD 1.1.1)

Seq. ID NO 5: Sequence of PDGB from Spleen (PBGD 1.3)

Seq. ID NO 6: Sequence of PDGB from bone marrow (PBGD 2.1)

Seq. ID NO 7: Sequence of PDGB from bone marrow (PBGD 2.2)

Seq. ID NO 8: Sequence of PDGB from lymph node (PBGD 3.1)

Seq. ID NO 9: Sequence of PDGB from lymph node (PBGD 3.3)

7.A II. Development of lab scale fermentation

The overall strategy for the development of the fermentation process was outlined as follows. The use of a minimal medium in the inoculum steps should facilitate the stability of the host, and a minimal medium supplemented with yeast extract and peptone should facilitate growth and production in the main fermentation. To reach high cell densities a concentrated glucose feed was used to control the growth rate in the feed phase. In the expression plasmid pExp1-M2-BB the *rop* gene has been deleted [8] which means that the expression of rhPBGD can be temperature regulated. Initially it was decided to start with a fermentation temperature of 30 °C, which means that no temperature induction was used. If the productivity at this temperature was unsatisfactory the temperature could be increased to 37 °C or 42 °C to increase the productivity. Oxytetracycline was chosen as selection pressure, but if possible with regard to plasmid stability, the main fermentation should run without any selection pressure at all.

7.A.1 Initial batch experiments

The study was initiated 1/2/99 and the intermediary strain PBGD-1 was delivered 4/2/99. An initial M9H-Tc (6 mgl^{-1})(attachment 2, table 2) shake flask cultivation (PD03) was performed to study the growth in the M9H-Tc (6 mgl^{-1}) inoculum medium recommended by Icogen Inc. A fermenter medium designated MM5Y-Tc (6 mgl^{-1}) was designed based on BioGaia Fermentations know how from other recombinant *E.coli* fermentations. This medium was first tested in a shake flask cultivation (PD04) before two 1 L batch fermentations (PD05 and PD06) were performed with two variants of the medium. PD06 was performed in MM5Y-Tc (6 mgl^{-1}) substrate complemented with 2 gl^{-1} tryptone (table 34) to investigate if tryptone could facilitate growth .

In a batch cultivation exponential growth continues for a relatively few generations until nutrients are depleted or toxic products accumulates. Due to this growth begins to slow and thereafter the micro organisms enter the stationary phase, where a steady state cell number is reached.

In all these batch experiments the initial glucose concentration was 10 gl^{-1}. The parameters defined below were analysed or calculated, and the results are summarised in table 34 below.

Maximum growth rate (μ_{max}) $dX / dt = \mu \times X$, where X = Dry cell weight

7.A.3 III. Scale up of fermentation

The scale up part of the development was divided into two parts. First simulated large scale fermentations were performed in laboratory fermenters to study the effect of the increasing number of generations on plasmid stability and product quality. These tests were followed by the actual 850 L fermentations to investigate the effects from increasing the fermentation scale on plasmid stability and product quality.

BioGaia Fermentations 1500 L production fermenters are normally inoculated with broth from one 14 L fermenter. To mimic the fermentations PD14-PD19 the OD_{620} after inoculation should be approximately 0,1. When using a working volume of 9 L in the he 14 L fermenter the final OD_{620} necessary to achieve the same inoculum conditions in 850 L can be calculated as follows

$OD_{620} \times 9\ L = 0{,}1 \times 850\ L \rightarrow OD_{620} = 9{,}4$

Inoculating the 14 L fermenter containing 9 L substrate with 500 ml broth (two 1 L shake flasks) with an OD_{620} of approximately 1,0 gives an OD_{620} initial of approximately 0,1-0,2. With a growth rate of 0,4 h^{-1} it will take 9-11 h to reach an OD_{620} of about 9. Since a growth rate of 0,4 h^{-1} is equivalent to a generation time of 1,7 h (ln2/0,4) this corresponds to 5-6 extra generations compared to the earlier lab scale fermentations.

An estimation of the number of generations in different steps of the entire process is given in table 37 below. The extra inoculum fermentation increases the total number of generations in the process with 15-25 %, an increase that could have effects on plasmid stability and product quality.

Table 37. Estimated number of generations in different process steps

Process step	Estimated number of generations
M9H-Tc agar plates	8 - 12
0,25 L M9H-Tc Shake flasks	4 - 5
9 L Inoculum fermentation	5 – 6
850 L Main fermentation	9 - 11
Σ Total process	26 - 34

Pressure Bar	Number of passages	Yield Total protein mg/ml	Yield Activity U/ml	Specific activity U/mg protein
800	1	1,2	4	3,3
600	3	1,6	7	4,3
800	3	3,5	16	4,6
1000	3	1,7	10	5,9

Exchanging the micro filter (0,2 µm) for ultra filters (500 K and 1000 K) resulted in less fouling at the filter surface and protein concentration increased to acceptable yields (Table 41). The smaller membrane area and flatter surface minimised product hold up and adsorption, which in this case increases yields. Furthermore, the transmission of total protein seems to be lower than the transmission of rhPBGD when using an ultra filter (1000 K), resulting in a higher specific activity (table 41). In all experiments the homogenate was dia filtered to the same theoretical yield (90%) to enable comparison of the results.

Final statement: For scaling up to production scale a 1000 K filter was chosen for cell debris removal since this filter gave a good yield of rhPBGD with a high specific activity. Diluted homogenate was decided to be concentrated approximately 2,5 times and then dia filtered with 50 mM sodium-phosphate, 1,34 mM EDTA, pH 7,4 to get a theoretical yield of rhPBGD of about 95 %. Nitrogen was flushed over the permeate surface in order to prevent oxidation (PD1502). Permeate-flux was set at 15 l $m^{-2}h^{-1}$ and the temperature was set at 15- 25°C (PD1501-PD1502).

Table 42. Summary of rhPBGD yield from Batch PD21 using different filters during cell debris removal. Homogenisation parameters: 800 bar, 3 passages. The yield of rhPBGD from membrane filtration was compared to centrifuged material.

Cell debris removal by	Yield Total protein mg/ml	%*	Yield Activity U/ml	%*	Specific activity U/mg protein
0,2 µm filter	2,2	30	7	21	3,3
500 K filter	4,8	65	22	69	4,6
1000 K filter	3,1	42	21	66	6,8
centrifuged	--	80	--	90	5,1

* Yield is given in % compared to homogenate.

7.A.4.4 Final filtration

Membrane filtered extracts contained less particles and was thereby easier to filter than centrifuged extract, where problems with clogging on the filter surface occurred. The clogging made it difficult to perform integrity tests. A white slippery precipitate was always seen in extract before the final filtration. When analysing the dissolved precipitate spectrofotometrically at OD_{260} /OD_{280} resulting in a ratio near 2, it was concluded that it contained nucleic acid.

7.A.4.5 Scale up of down stream process (PD22, PD1501 and PD1502)

The entire final process was at first tested at a 15 L scale (batch PD22) ending with a rhPBGD yield of 75 %. When scaling up to 300 L(PD1501) problems with precipitation in the broth occurred and the rhPBGD yield decreased to 46 %. When processing batch PD1502 no precipitation was seen and the rhPBGD yield increased to 77 %. The results are summarised in tables 43 and 44.

The low yields of rhPBGD from batch PD1501 was probably due to several factors: Using the same filter unit at cell concentration and cell debris removal commonly saves both money and time but when a white precipitate occurred in the broth it resulted in problems cleaning the filter between cell concentration and cell debris removal. The composition of the white insoluble precipitate was analysed and the results are shown in table 43. To avoid the precipitate formation in PD1502 the substrate preparation was carefully monitored. No new component was added until the former component was completely dissolved. No precipitate was formed in batch PD1502.
The filter area was small in comparison to the processed volume, which increased the chances for clogging on the filter surface. Dia-filtration was only performed to achieve a theoretical yield, rhPBGD, of 90 %.

In batch PD1502 the concentration of rhPBGD in the extract was low compared to batch PD22 but the yield was slightly higher. The lower concentration was due to an operator mistake using an increased dia filtration volume at cell debris removal during the process of PD1502. If a smaller dia filtration volume during cell debris removal in batch PD1502 had been used it would have resulted in a higher concentration of rhPBGD but the yield had then decreased.

at 72°C was used at the end to ensure that the extension products were filled out. One of this PCR mix was again amplified exactly as described above and cloned into pBluescript II SK- (Stratagene, catalogue # 212206), linearized with *Eco*R I and *Hin*d III after purification (using GEANECLEAN III, from BIO 101 catalogue # 1001-600) and digestion with the same two enzymes (see Figure 37, A and B).

8.2 Sequencing

Four plasmid clones from the above ligation viz. pBlueAlaD-1-4 were sequenced with the Big Dye terminator cycle sequencing kit from PE/ABI catalogue # 4303152. Three vector primers, ICO383 (5' GTAATACGACTCACTATA GGGC 3'), ICO384 (5' CTAAAGGGAACAAAAGCTGGAG 3') and IC0618 (5- GCGCGTAATACGACTCACTA 3) and two ALAD-specific primers, ICO618 (5' CCTACGCTGTGTCTTGATCT 3') and ICO617 (5' GGCTT CACCATGAGCATGTC 3') were used. The results are tabulated in Table 46.

Table 46 Summary table of sequencing results

Clone #	Nucleotide change	Amino acid Change
BlueAlaD-1	-	-
BlueAlaD-2	-	-
PBlueAlaD-3	168, T to C 414, C to T 463, T to C 866, C to T	56, Y (silent) 138, N (silent) 155, L (silent) 289, A to V
PBlueAlaD-4	180, T to C	56, y (silent)

Reporting and Results

The inserts in all four clones confirmed to be ALAD by sequence analysis. The results are shown in Table 46 As seen, two of the clones completely match the published sequence (2). The other two have changes, most of which are silent. Without a larger sampling volume it is difficult to distinguish between allelic variation and PCR/cloning artifacts. The

ALAD insert from pBlueAlaD-2 was used for expression purposes and its sequence is shown in seq. 14.

Evaluation and conclusions

The PCR amplification strategy used has generated ALAD cDNA that matches the published sequence. It has convenient restriction sites at the ends for ease of manipulation into expression vectors, including an engineered BsrD I site just upstream of the ATG.

Example 9

Administration for rhPBGD, an animal study.

Recombinant human Porphobilinogen Deaminase (rhPBGD) will be administered as an enzyme substitution treatment for patients diagnosed with Acute Intermittent Porphyria (AIP). rhPBGD will be administered by s.c or i.v injections. It is essential for the efficacy of the treatment, e.g. reduction of the toxic precursors porphobilinogen (PBG) and δ-aminolevulinic acid (ALA), that rhPBGD can enter the blood stream and remain biologically active.

Pharmacokinetics of rhPBGD

To study the pharmacokinetics, wildtype healthy B6 mice were injected with rhPBGD. The content and enzyme activity of rhPBGD was followed in plasma from animals after different timepoints (0, 15, 30, 45 and 60 min). Each animal received one single injection of 50 µg rhPBGD and three different routes of administration were used, i.v, i.p or s.c. The plasma levels of rhPBGD analysed by ELISA are shown in Figure 1. The conclusion from this data is that the half-life of rhPBGD following i.v injection is 20-30 min. Following i.p injection the maximal levels of rhPBGD was found after approximately 30 min. Also s.c injections of rhPBGD resulted in detectable levels of PBGD in plasma which shows that it is possible to use this route for administration. S.c injection did also result in a slow release of rhPBGD to plasma with maximal levels found in the last timepoint analysed (60 min).

FIGURE 38 shows plasma levels of rhPBGD following administration to mice. 50 µg rhPBGD (2,3-2,8 mg/kg) were injected i.v, i.p or s.c to wildtype B6 mice. After different timepoints (0, 15,

In this study all animals were treated with an increasing dose of phenobarbital for four days (day 0-4, 75-90 mg/kg and day i.p). One group of animals did at the same time also receive rhPBGD for seven days (day 0-7, 1,7-2,3 mg/kg and day i.p). The content of PBG and ALA were analysed in 24 h urine samples where levels are expressed as mmol/mol creatinine. As seen in Figure 41 the rhPBGD treatment results in a lowering of urinary content of PBG and ALA as compared to animals treated with only phenobarbital (Figure 40). This data shows that rhPBGD, when given to mice with high levels of PBG and ALA in serum (acute AIP attack), can lower these levels as analysed by the urine content of this metabolites. No antibody formation against rhPBGD was seen in these animals when analysing at day 11 or at 2 weeks after that the rhPBGD treatment was stopped.

The conclusion from this data is that rhPBGD can lower the levels of PBG and ALA in mice during an acute attack of porphyria. This may also indicate that clinical symptoms seen in AIP patients, which probably are induced by the high serum levels of PBG and ALA, may be reversed by this treatment. Further studies are now on its way to confirm this data. It is also possible to increase the treatment period using rhPBGD in mice due to that no antibody formation was seen.

Fig 40 shows the urinary content of PBG and ALA in AIP-mouse treated with phenobarbital. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4, 75-90 mg/kg and day i.p). PBG and ALA levels were analysed in 24-h urine samples and expressed as mmol/mol creatinine. Data from one representative animal are shown.

Fig. 41shows the urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4, 75-90 mg/kg and day i.p) and rhPBGD for 7 days (day 0-7, 1,7-2,3 mg/kg and day i.p). PBG and ALA levels were analysed in 24-h urine samples and expressed as mmol/mol creatinine. Data from one representative animal are shown.

Ongoing studies in mice

Known clinical symptoms in AIP patient are different neurological symptoms such as pain in stomach and/or legs and arms and muscle weakness. To study these symptoms in the transgenic mice we also analysed the motorneuron function by different behavioural tests such as rotarod and grip strength. Data shows that the transgenic AIP-mouse have

significantly lower activity in all behavioural tests as compared to wildtype controls. See examples of such data from the grip strength (Figure 42) and from rotarod (Figure 43). Motor neuropathy has also been described in the AIP-mice by Lindberg, R. L. P. et al. Journal of Clinical Investigation 103:1127-1134, 1999. We will now analyse if also the neurological disorders in the AIP-mouse can be can be reversed by the rhPBGD treatment, as a possible long-term treatment for patients.

Fig 42 shows the grip strength analysis in control and AIP-mice. Grip strength were determined using a grip strength meter (Ugo Basile) in heterozygous control animals (control 1, n=5), in wild type controls (control 2, n=5) and in AIP-transgenic mice (AIP, n=5).

Fig. 43 shows a rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Example10

Stability data for rhPBGD

Stability study 1 - Selection of formulation buffer

To find the best suitable formulation conditions for rhPBGD-His, the enzyme was formulated in a phosphate buffer containing mannitol and glycine as protein stabilizers. Different pH, ion strength and enzyme concentration were investigated, Table 47. The study was performed at 40°C and 75% relative humidity during 8 weeks.

Table 47. Formulations

Sample no.	pH	Ion strength (mM)	rhPBGD-His conc. (mg/ml)
1a, 2c, 3a	7,5	10	2
1b	7,5	10	8
2a	6,5	10	2
2b	7,0	10	2
2d	8,0	10	2

2e	8,5	10	2
3b	7,5	50	2
3c	7,5	100	2

The samples were sterile filtered and aliquots of 170 l were dispensed into 300 l glass vials with a Teflon/silicon crimp cap. Samples were collected for analysis on week 0, 1, 2, 4 and 8 and were analysed for enzyme activity (enzyme activity assay), protein concentration (HPLC) and degradation/aggregation (HPLC, SDS-PAGE and IEF).

The enzyme precipitated in all samples except for sample no. 2e (pH 8,5). Sample no. 1b (8 mg/ml) showed a higher precipitation rate than samples with lower enzyme concentration. This sample turned yellow after one week, all other samples turned yellow after 2 weeks. The HPLC chromatograms showed that a prepeak to the rhPBGD-His peak was formed during storage at 40°C, and that this prepeak increase with time. The peak for pure rhPBGD-His decreased with time. Figure 1, shows the amount enzyme calculated from the area under the peak in HPLC chromatograms.

The enzyme activity, Figure 45, decreased from 16 Units/ml (U/ml) to 2 U/ml in 8 weeks, for sample no. 1b the decrease in enzyme activity was more pronounced the first week, 40 U/ml to 10 U/ml, corresponding to the precipitation seen in Figure 44. The specific enzyme activity is shown in Figure 46. It seemed like high protein concentration was detrimental for the enzyme activity (sample no. 1b).

SDS-PAGE gels showed bands from aggregates as well as from scissoring after one week for all samples.

On the isoelectric focusing, IEF, gels four bands corresponding to the four catalytic forms of the enzyme (E, E1, E2 and E3) were seen on day 0. During storage at 40°C it seemed like the first band (E) was weakened and the second band (E1), which is more acidic was getting stronger. The number of more acidic bands increased over time. This was probably due to deamidation.

Conclusion

rhPBGD-His was not stable at 40°C. However, the only formulation buffer in which no visible precipitation of the enzyme was detected was no. 2e (pH 8,5). High concentration of

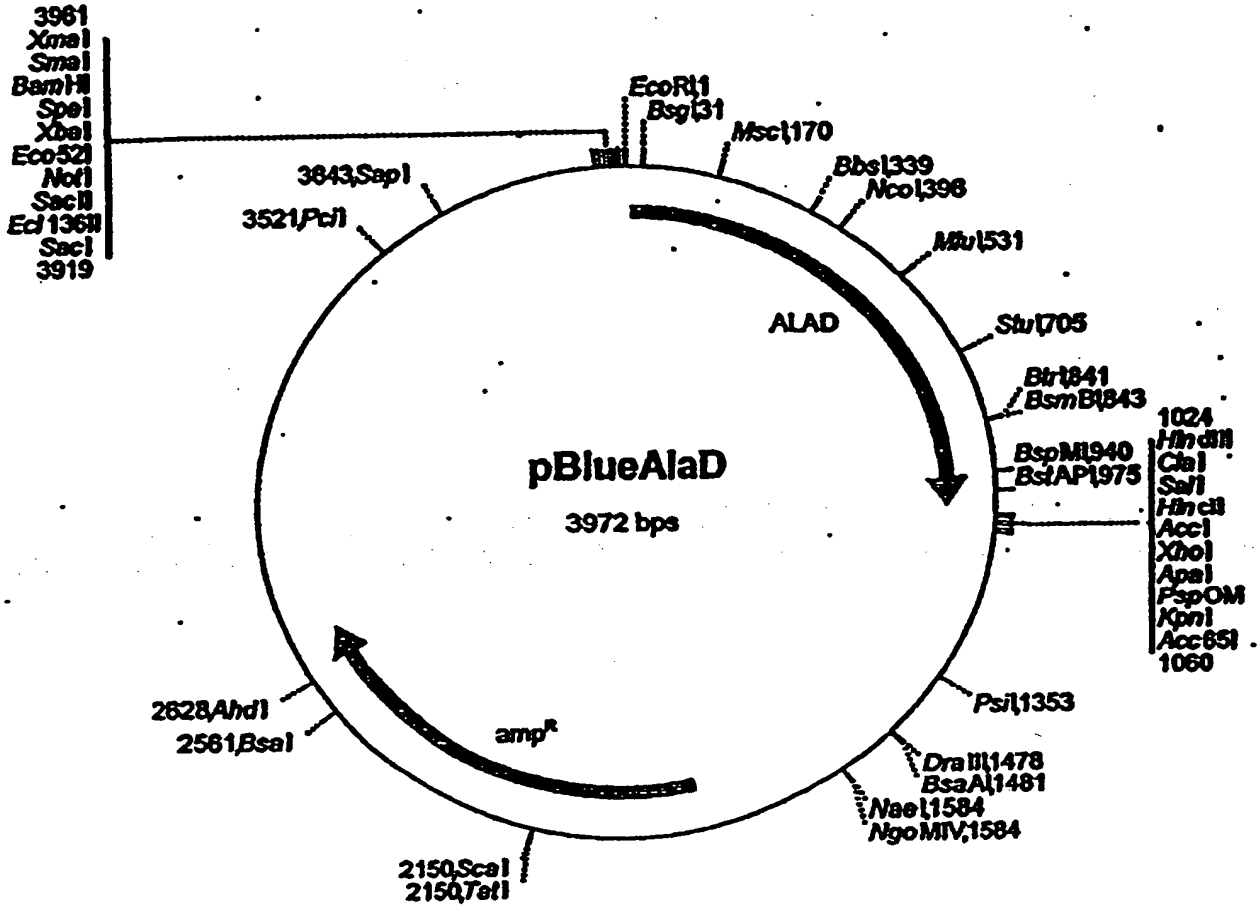

Fig. ~~38~~ 37C

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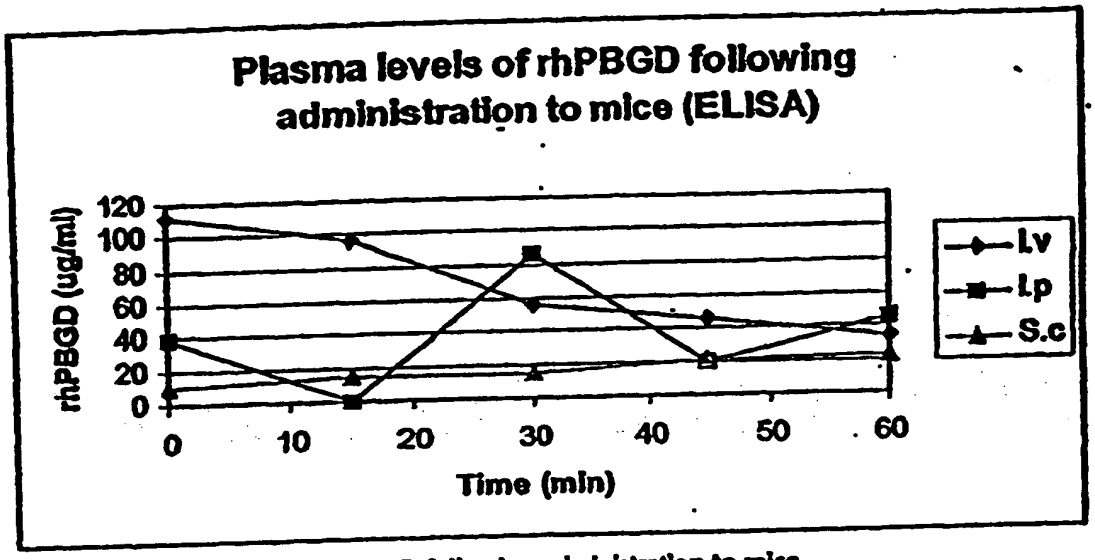

Figure 38: Plasma levels of rhPBGD following administration to mice.

~~Fig. 39~~

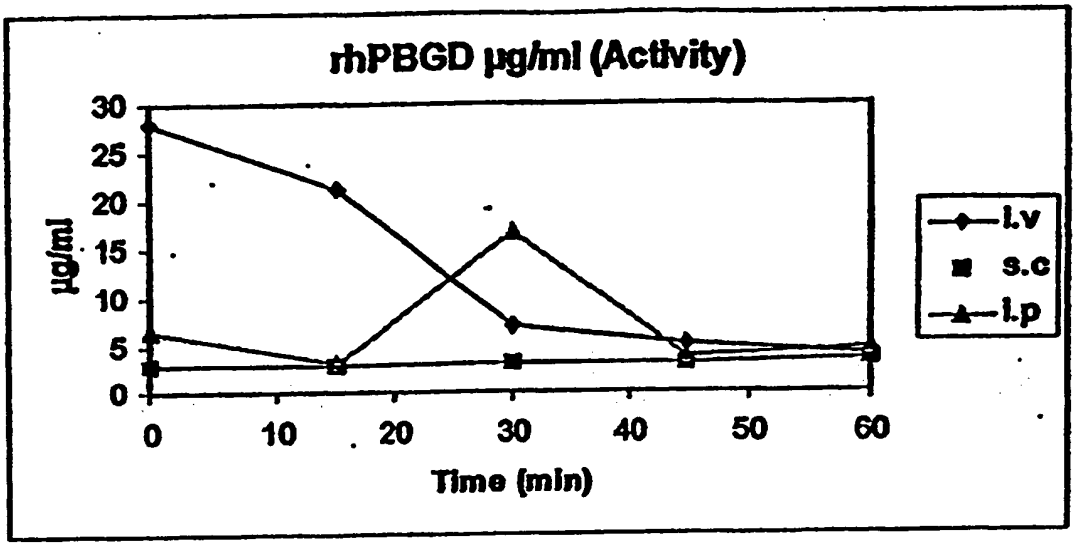

Figure 39: PBGD enzymatic activity in plasma following rhPBGD administration to mice

Fig. 40

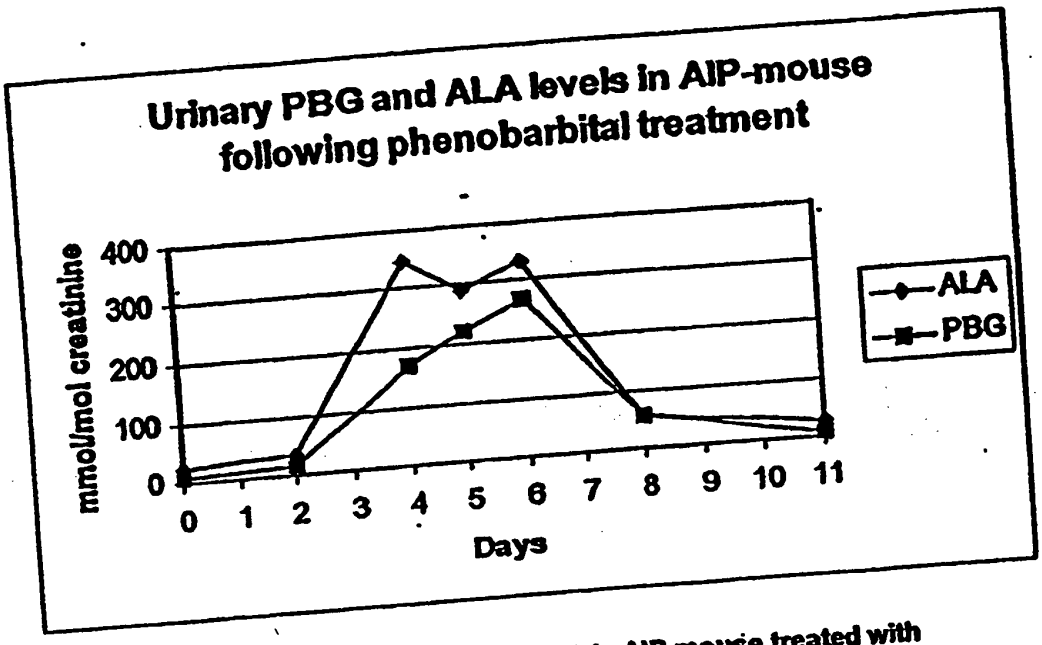

Figure 40: Urinary content of PBG and ALA in AIP-mouse treated with phenobarbital.

~~Fig. 41~~

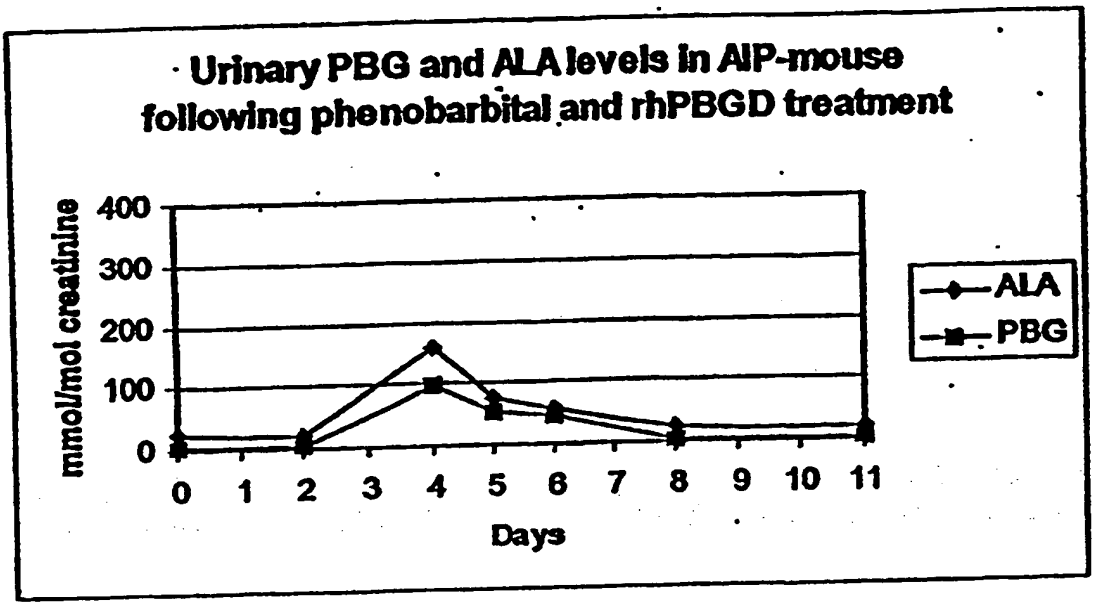

Figure 41: Urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4,

Fig. 42

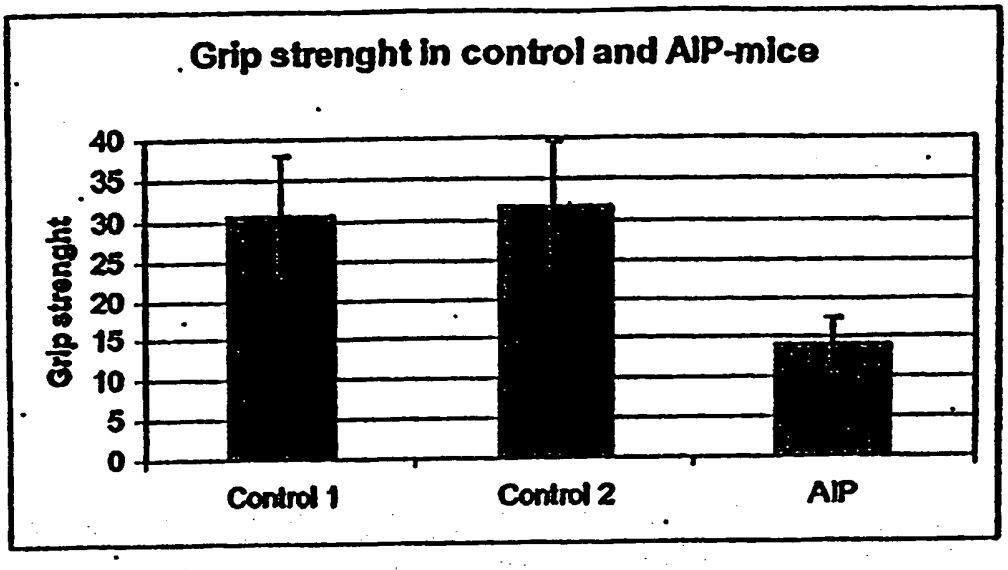

Figure 42: Grip strength analysis in control and AIP-mice. Grip strength were determined using a grip strength meter (Ugo Basile) in heterozygous control animals (control 1, n=5), in wild type controls (control 2, n=5) and in AIP-transgenic mice (AIP, n=5).

Fig. 43

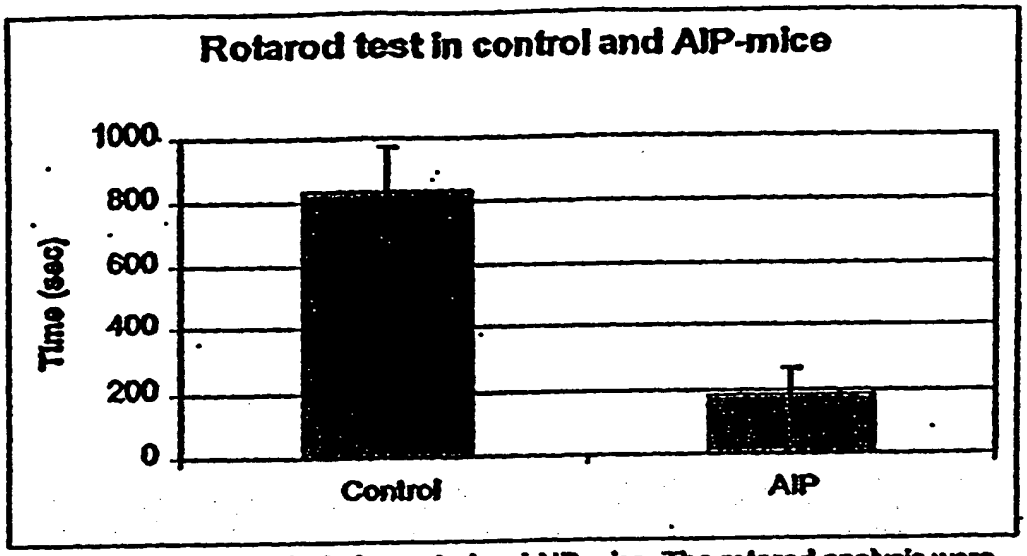

Figure 44 Rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Fig. ~~44~~ 43

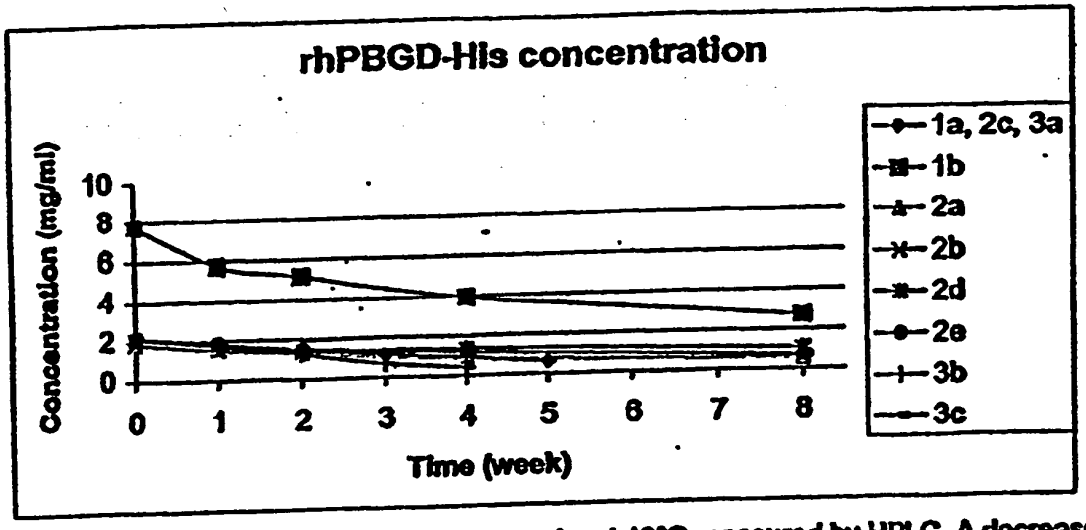

Figure 44. Enzyme concentration over 8 weeks at 40°C measured by HPLC. A decrease from 2 mg/ml to 0,5 mg/ml and 8 mg/ml to 2,5 was detected.

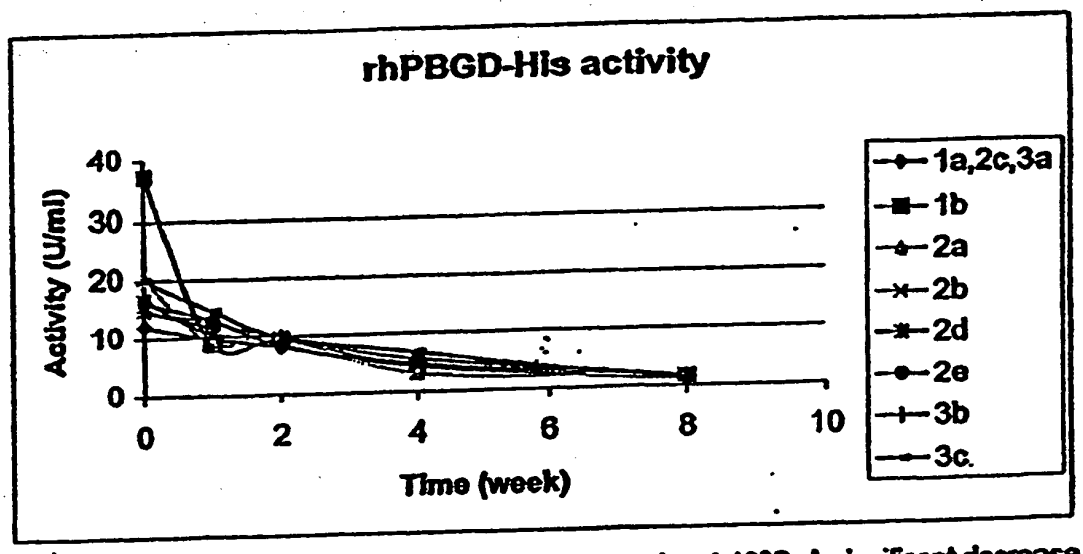

Figure 45. The enzyme activity measured over 8 weeks at 40°C. A significant decrease over the first week was seen for the high concentration sample, 1b. After two weeks the decrease rate was the same for all samples.

~~Fig. 45~~

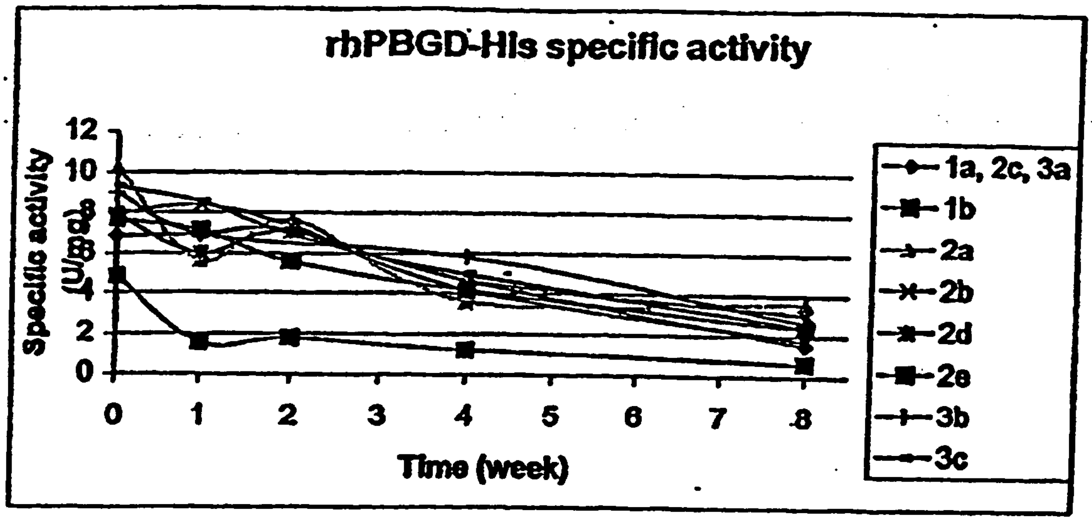

Figure 46. Enzyme specific activity measured during 8 weeks at 40°C. The activity was measured using the enzyme activity assay and the protein concentration was measured using HPLC.

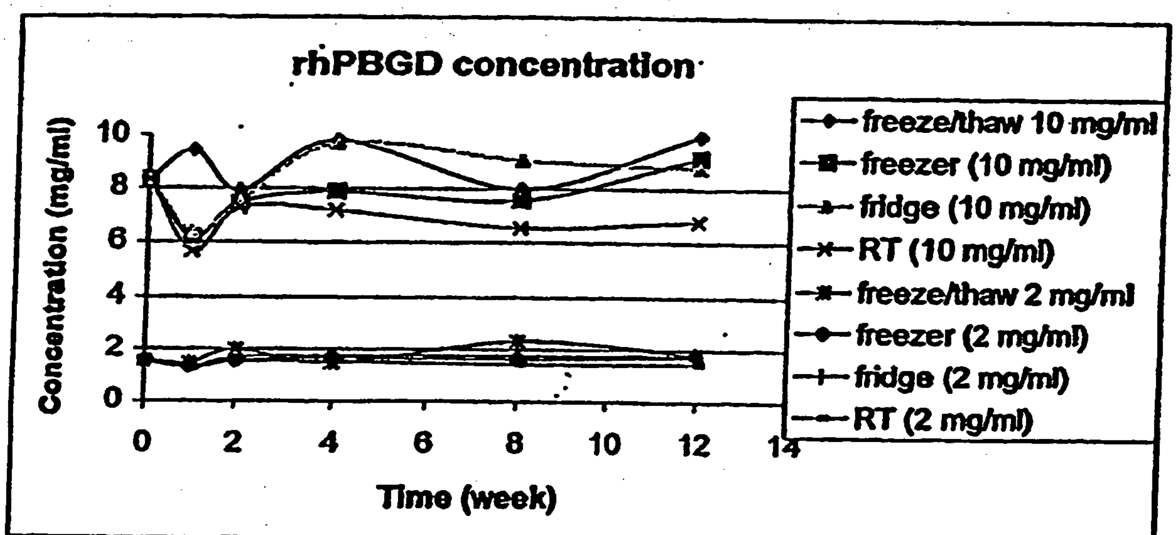

Figure 47. rhPBGD concentration over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. The measurement was performed using HPLC.

Fig. 46

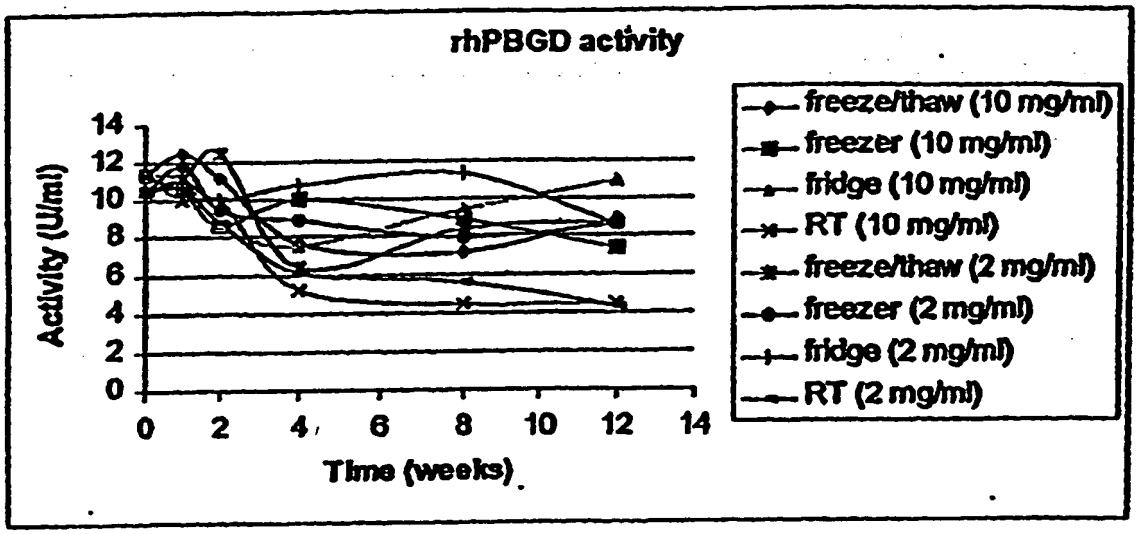

Figure 48. rhPBGD activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling.

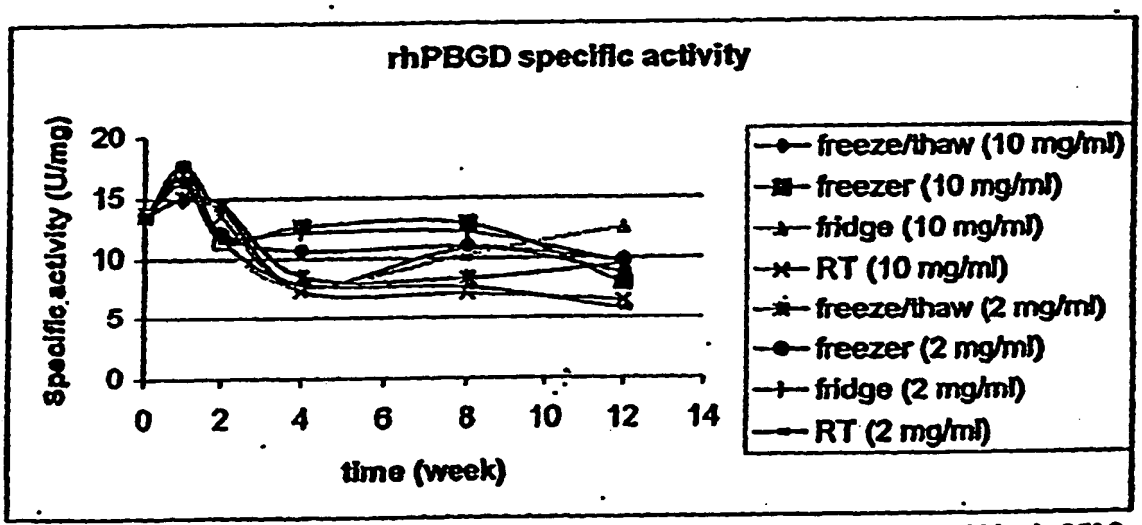

Figure 49. rhPBGD specific activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. Measurements were performed using enzyme activity assay and HPLC.

~~Fig. 47~~

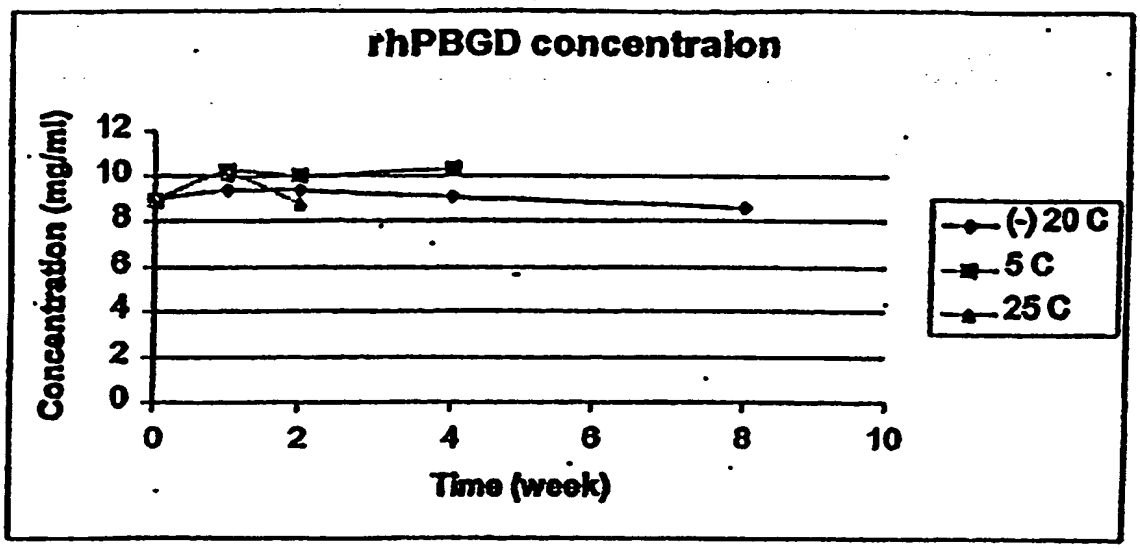

Figure 50. rhPBGD concentration measured over 8 weeks using BCA.

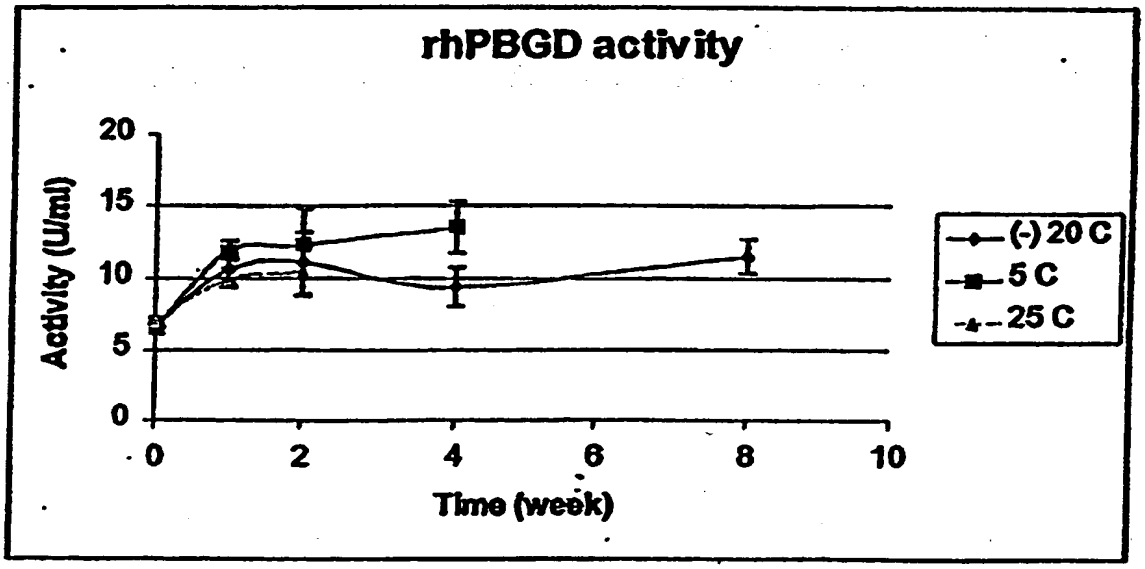

Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at -20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Fig. 48

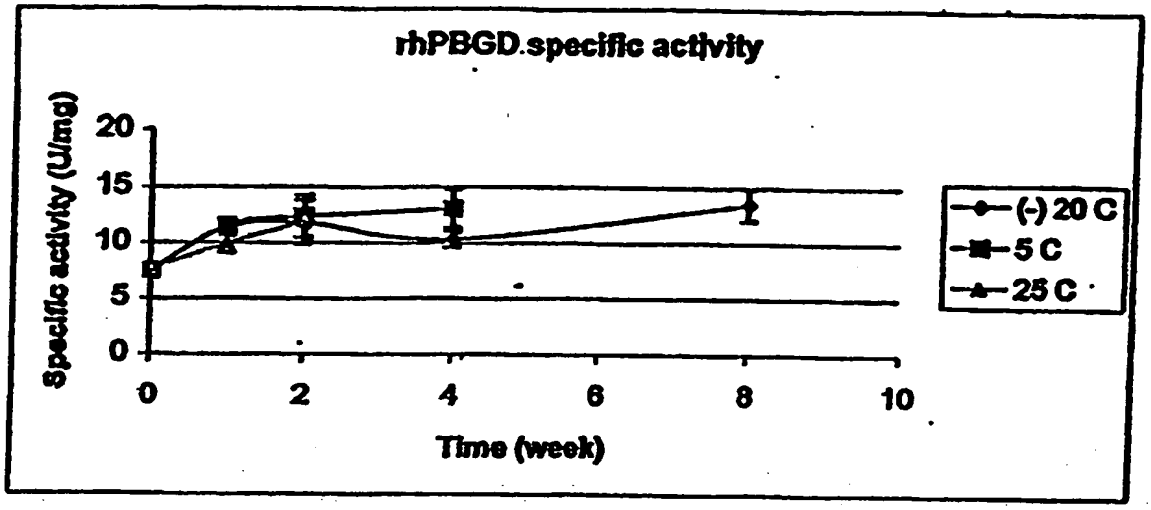

Figure 52. The specific rhPBGD activity measured using the enzyme activity assay and BCA protein concentration assay. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Fig. 49